The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

INVESTOR IN PEOPLE

CERTIFIED COPY OF
PRIORITY DOCUMENT

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed D. Mahoney

Dated 13 August 2004

21NOV02 E765257-1 C86721
P01/7700 0.00-0227179.9

1/77

THE PATENT OFFICE
A
21 NOV 2002
RECEIVED BY FAX

Patents Form 1/77

Patents Act 1977

(Rule 16)

The Patent Office

The Patent Office

Cardiff Road
Newport
South Wales
NP10 8QQ

Request for grant of a patent

(See the notes on the back of this form. You can also an explanatory leaflet from the Patent Office to help you fill in this form)

1. Your reference — IM0030 — P3576OWO | NJL.

2. Patent application number *(The Patent Office will fill in this part)* — 0227179.9 — 21 NOV 2002

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

UNIVERSITY OF LEICESTER
DEPT. OF MEDICINE & THERAPEUTICS
CLINICAL SCIENCES BUILDING, LEICESTER, LE2 7LX
UNITED KINGDOM

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation — **UNITED KINGDOM** — 00798348003

4. Title of the invention — **BODILY FLUID MARKERS OF TISSUE HYPOXIA**

5. Name of your agent *(If you have one)* — Nicholas John Lee Kilburn & Strode — **ANDREWS, Martyn P** — Kilburn & Strode 20 Red Lion St London

"Address for Service" in the United Kingdom to which all correspondence should be sent *(including the postcode)* — 20 Red Lion St London WC1R 4PJ — **PATENT DEPARTMENT, UNIPATH LTD**
PRIORY BUSINESS PARK
BEDFORD, MK44 3UP
WC1R 4PJ 125001

Patents ADP number *(if you know it)* — 125001 — ~~08488841600~~

S1/77 7/7/04 S1/77 21/11/03

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day/month/year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

YES

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	
Description	14
Claim(s)	1
Abstract	-
Drawing(s)	12

10. If you are also filing any of the following, state how may against each item.

Priority Documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	2 pages of references 1 page entitled Table 1.

11. I/We request the grant of a patent on the basis of this application.

Signature(s) Isobel Howell Date: 21/11/02

Isobel HOWELL

12. Name and daytime telephone number of person to contact in the United Kingdom — Isobel Howell, Tel 01234 835482

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) *If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.*

b) *Write your answers in capital letters using black ink or you may type them.*

c) *If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.*

d) *If you have answered 'Yes' Patents Form 7/77 will need to be filed.*

e) *Once you have filled in the form you must remember to sign and date it.*

f) *For details of the fee and ways to pay please contact the Patent Office.*

Title: Bodily fluid markers of tissue hypoxia

Field of the Invention

This invention relates to the detection and measurement in a bodily fluids of markers of tissue hypoxia or of a clinical syndrome attributable to tissue hypoxia in a mammalian subject. In particular this invention relates to the identification of Oxygen Regulated Protein (ORP150) or peptide fragments derived thereof as useful cardiac or vascular disease markers and also to their use as such in an immunoassay. This marker may be used alone or in combination with additional markers.

Background to the Invention

Ischaemic heart disease and heart failure are major health problems in the world but the means to effectively diagnose and manage these conditions at the moment are limited.

Chronic heart failure

Chronic heart failure (CHF) is a common clinical syndrome which is an increasingly important health care issue in industrialised societies with elderly populations. Hospitalisation rates for heart failure increased markedly over the last 20 years and CHF is associated with poor prognosis and quality of life. The direct costs of CHF account for approximately 1-2% of health care expenditure, the vast majority being related to hospital admissions.

Chronic heart failure is most often the result of left ventricular systolic dysfunction (LVSD). Screening studies from Glasgow [1] and Birmingham [2] indicated prevalence of rates of definite LVSD of 2.9% and 1.8% respectively. In both studies, the condition was asymptomatic in half of the cases. The identification of patients with LVSD allows the prescription of appropriate therapy which for the individual patient improves quality of life and prognosis. Echocardiography is currently the most frequently used investigation for the diagnosis of LVSD and heart failure.

The pathophysiology of CHF involves activation of many neurohormonal systems, including the catecholamine, renin-angiotensin, endothelin, atrial and brain natriuretic peptide systems. Some of these systems are activated in an adaptive fashion (the natriuretic peptide systems); others although adaptive under acute conditions become maladaptive especially when maintained in the chronic state (endothelin, renin-angiotensin and catecholamine systems). An increased secretion of the natriuretic peptide hormones has been exploited as a means for diagnosis of CHF [3,4]. For detection of LVSD, brain natriuretic peptide (BNP-32) is a better diagnostic tool than N-terminal pro-atrial natriuretic peptide (N-ANP) [3]. In addition, another peptide derived from the precursor of BNP-32, namely N-terminal proBNP (N-BNP) is also a reasonable alternative for the identification of LVSD [4]. In both cases, the negative predictive values of the tests are high, suggestive of their utility in the exclusion of CHF. In many cases of CHF, the aetiology is ischaemic heart disease. In addition, a reduced cardiac output over a chronic period would lead to tissue hypoperfusion and a relative tissue hypoxia.

However, an indicator in the plasma that is induced and secreted when tissues are hypoxic would have great utility in the diagnosis and prognosis of heart disease, and may have further utility in the monitoring of such diseases.

Ischaemic heart disease

Ischaemic heart disease is a major health burden in developed countries, and its main aetiology is atherosclerosis. Accumulation of lipid, especially oxidised or modified LDL, together with macrophages and other cells leads to plaque growth and instability.

Oxygen Regulated Protein ORP150 and its role as a molecular chaperone in the Endoplasmic Reticulum

Oxygen regulated protein (ORP150) is a chaperone endoplasmic reticulum associated protein that was originally cloned from astrocytes subjected to hypoxia [5]. The induction of this protein in rat astrocytes, human aortic vascular myocytes and mononuclear leucocytes showed specificity for hypoxia but not other stressful stimuli such as glucose deprivation, hydrogen peroxide, tunicamycin or heat shock [5,6]. In addition, tissue extracts prepared from human atherosclerotic lesions demonstrated increased expression of ORP150 mRNA and protein, with most of the mRNA found in macrophages [6]. ORP150 may function as a molecular chaperone in cells of the atherosclerotic vessel wall, present mainly in the endoplasmic reticulum (ER) of cells subjected to hypoxia [6]. Its expression may be further enhanced by the presence of oxidised or acetylated light density lipoproteins (LDL), a known risk factor for vascular disease [6]. Its functions include a protective effect against hypoxia induced damage, since reducing its expression with antisense oligonucleotides leads to enhanced susceptibility of mononuclear phagocytes to hypoxic damage [6].

The cloning of human ORP150 recently demonstrated a deduced amino acid sequence of 999 residues, with an endoplasmic reticulum (ER) retention signal at the C terminus [7]. It has some homology to other stress induced proteins such as glucose regulated protein (GRP170) and heat shock protein (HSP70). Its location in the ER suggests a role as a molecular chaperone for protein folding and maturation, especially in hypoxic conditions. For example, kidney cells ORP150 antisense transformants showed delayed maturation of glycoprotein GP80, the latter accumulating in the ER, thus indicating a role for ORP150 in protein maturation and transport within ER of cells under conditions of energy deprivation [8].

Evidence for a function of ORP150 in vascular disease comes from observations on a stroke model in mice [9,10]. In a mouse model of cerebral ischaemia, there was rapid induction of ORP150 mRNA and protein in the hypoxic neurons, even within the ischaemic and energy depleted zones [9]. In ischaemic human brains, although ORP150 expression in neurons was only sparingly induced, there was a significant induction of ORP150 in astrocytes [10]. Neurones overexpressing ORP150 were resistant to hypoxic stress and mice genetically engineered to overexpress ORP150 in their neurons had smaller strokes under ischaemic stress [10]. Cytoprotection was associated with suppressed caspase-3-like activity and enhanced brain-derived neurotrophic factor (BDNF), indicating a role for ORP150 in cytoprotection under hypoxic conditions [10]. In addition, astrocytes or cell lines transfected to overexpress ORP150 antisense RNA were more susceptible to hypoxic stress, leading to apoptosis [10,11].

Other evidence supporting a role for ORP150 in vascular disease lies in its association with angiogenesis [12-14]. ORP150 is expressed in tumours, with high expression in the cells invading host tissue [10]. Invasion is often associated with the co-expression of vascular endothelial growth factor (VEGF, a recognised angiogenic factor) and ORP150 [13], and tumour cells transfected with antisense ORP150 are less invasive [13], with defective maturation of VEGF leading to its accumulation in the ER [13]. In wounds, neovascularisation is associated with expression of both VEGF and ORP150 [14]. Local administration of adenovirus bearing ORP150 leads to enhanced wound repair, new vessel formation and VEGF expression at the site [14]. Macrophages engineered to reduce ORP150 expression have defective VEGF maturation, the latter accumulating in the ER, whereas overexpression of ORP150 leads to successful export and secretion of the VEGF product [14]. Enhanced angiogenesis with ORP150 expression would further support its role a cytoprotective agent from its other intracellular effects on apoptosis suppression under hypoxic stress.

Summary of the invention

In conditions of tissue hypoxia, there may be increased expression of ORP150 in tissues. However, the ORP150 protein is associated with endoplasmic reticulum and is not expected to be secreted. However the present inventors have shown that ORP150 or peptide fragments derived from ORP150 can be detected in human plasma or other bodily fluids such as urine, whole blood or serumusing an immunoassay and have been able to show its utility as a marker for the diagnosis and prognosis of CHF.
Furthermore ORP150 or peptide fragments thereof have been detected in plasma at higher levels in patients who have heart failure and ischaemic heart disease (e.g. myocardial infarction) than normal subjects. Measurement of these markers may therefore be useful as a diagnostic aid for presence of heart failure and for assessing the severity of heart failure.

ORP150 or peptide fragments thereof may also be useful in assessing the prognosis of patients with ischaemic heart disease or acute coronary syndromes; especially after myocardial infarction, levels of the peptide are elevated in patients at risk of increased mortality or readmission with heart failure.

ORP150 or peptide fragments thereof may be useful in combination with the natriuretic peptides (e.g. N-terminal proBrain natriuretic peptide or N-BNP) in assessing the prognosis of patients with ischaemic heart disease or acute coronary syndromes; after myocardial infarction, the combination of peptides is useful in risk stratification of patients with respect to mortality.

Uses of ORP150 in diseases associated with tissue hypoxia

ORP150 is hyperexpressed in the vessel wall of atheromatous plaques [6], present mainly in the macrophages. Since we have demonstrated increased ORP150 in plasma from patients with heart failure and ischaemic heart disease (of which the main cause is atherosclerosis), it is likely that patients with other clinical syndromes attributable to atherosclerosis such as ischaemic stroke, aortic aneurysm, peripheral vascular disease or other acute coronary syndromes may have increased ORP150 in their plasma, and the marker could be used as an indicator of the presence or severity of these conditions. Similarly, patients with chronic lung disease where tissues may be hypoxic would also be expected to have increased ORP150 in

their plasma,. Currently, there is no easy way of measuring pO2 in patients with lung disease, who for example are on home oxygen therapy, and a further use of ORP150 measurements could be in the monitoring of oxygen therapy in such patients.As invasive tumours may overexpress ORP150 [12,13,17] as a chaperone for vascular endothelial growth factor, there is also the possibility of its use as a tumour marker, especially of invasive tumours that may be relatively hypoxic in their central regions. Such use would extend to diagnosis and also, for the monitoring of treatment of these invasive tumours.

Such assays could be competitive or non-competitive immunoassays. Such assays, both homogeneous and heterogeneous, are well-known in the art, wherein the analyte to be detected is caused to bind with a specific binding partner such as an antibody which has been labelled with a detectable species such as a latex or gold particle, a fluorescent moeity, an enzyme, an electrochemically active species and so on. The presence or amount of analyte present is then determined by detection of the presence or concentration of the label.

Such assays could be carried out in the conventional way using a laboratory analyser or with point of care or home testing device such as the lateral flow immunoassay as described in EP291194

Brief explanation of the figures

Figure 1. A standard curve for the ORP150 peptide competitive immunoassay. A patient's plasma extract (solid circles joined by solid line) was diluted in two fold steps, showing parallelism with the standard curve. Two patients' urine extracts were also diluted in two fold steps (hollow triangles joined by dotted lines), again demonstrating parallelism with the standard curve.

Figure 2. Size exclusion chromatography with analysis of the fractions for ORP150. The points of elution of markers for 150 kd, 20 kD and 6.5 kD are indicated by arrows. Three peaks of immunoreactivity for ORP150 are evident at 150, approximately 7 and approximately 3 kD.

Figure 3. Box plots of log transformed plasma N-BNP and ORP150 levels in normal subjects, heart failure patients and patients with myocardial infarction.

Figure 4. Box plots of log transformed plasma N-BNP and ORP150 levels in normal subjects and heart failure patients of both gender.

Figure 5. Relationship of plasma N-BNP and ORP150 with severity of heart failure (as judged by the NYHA class) in males and females.

Figure 6. Receiver Operating Characteristic curve for diagnosis of heart failure, using N-BNP or ORP150 alone, and using the prognostic index derived from a logistic model with a combination of N-BNP and ORP150.

Figure 7. Relationship of plasma N-BNP and ORP150 to Killip class in patients after myocardial infarction.

Figure 8. Relationship of plasma N-BNP and ORP150 to left ventricular function as assessed by echocardiography in patients after myocardial infarction. Ventricular dysfunction is classified as normal, mild, moderate or severe impairment.

Figure 9. Comparison of the levels of N-BNP and ORP150 to the clinical outcome of death in patients after myocardial infarction.

Figure 10. Comparison of the levels of N-BNP and ORP150 to the clinical outcome of rehospitalisation with heart failure in patients after myocardial infarction.

Figure 11. Survival analysis of patients following myocardial infarction, stratifying patients as below or above the median value of plasma N-BNP or of ORP150.

Figure 12. Survival analysis of patients following myocardial infarction, stratifying patients as having both plasma levels of N-BNP and ORP150 below or above the median, and an intermediate group in which either peptide is above their respective medians.

Detailed description of the invention

Study Populations

120 heart failure patients were studied, all with echocardiographically confirmed left ventricular systolic dysfunction (left ventricular (LV) ejection fraction < 45%). A further 373 patients with myocardial infarction were also recruited. Acute myocardial infarction was defined as presentation with at least two of three standard criteria, i.e. appropriate symptoms, acute ECG changes of infarction (ST elevation, new LBBB), and a rise in creatine kinase (CK) to at least twice the upper limit of normal, i.e. >400 IU/L. 177 of the myocardial infarction patients were also investigated with echocardiography, with systolic function graded as normal, mild, moderate or severe impairment. Age and gender matched normal controls with LV ejection fraction >50%, were recruited from the local community by advertisement. All subjects gave informed consent to participate in the study, which was approved by the local Ethics Committee.

End Points in myocardial infarction patients

End-points were defined as all-cause mortality and cardiovascular morbidity (rehospitalisation with heart failure) following discharge from the index hospitalisation. Multivariate analysis for all endpoints other than death was performed after the censorship of those patients dying during follow up.

Blood Sampling and plasma extraction

In normal subjects and heart failure patients, 20mls of peripheral venous blood was drawn into pre-chilled Na-EDTA (1.5mg/ml blood) tubes containing 500 IU/ml aprotinin after a period of 15 min bed rest. In myocardial infarction patients, a single blood sample was taken between 72-96 hours after symptom onset. After centrifugation at 3000 rpm at 4°C for 15 min, plasma was separated and stored at −70 °C until assay. Prior to assay, plasma was

extracted on C_{18} Sep-Pak (Waters) columns and dried on a centrifugal evaporator. Some urine specimens were also collected from patients with heart failure. These were also extracted on C_{18} Sep-Pak (Waters) columns as above.

Assay of ORP150

A peptide corresponding to the N-terminal domain (amino acids 33-45) of the human ORP150 sequence (LAVMSVDLGSESM) [7] was synthesized in the MRC Toxicology Unit, University of Leicester. Amino acids 1-32 may represent a signal sequence for the protein and may not be present in the mature ORP150 protein. A rabbit was injected monthly with this peptide conjugated to keyhole limpet hemocyanin using maleimide coupling to a cysteine added to the C-terminal of the sequence. IgG from the sera was purified on protein A sepharose columns. The above peptide was also biotinylated using biotin-maleimide in buffer containing (in mmol/l) NaH_2PO_4 100, EDTA 5, pH 7.0 for 2 hours. After quenching with excess cysteine, the tracer was purified on HPLC using an acetonitrile gradient. Alternatively, the above peptide could be synthesized with incorporation of a biotinylated amino acid at the C- or N-terminus and used as a tracer. Plasma extracts and standards were reconstituted with ILMA (immunoluminometric assay) buffer consisting of (in mmol/l) NaH_2PO_4 1.5, Na_2HPO_4 8, NaCl 140, EDTA 1 and (in g/l) bovine serum albumin 1, azide 0.1. ELISA plates were coated with 100 ng of anti-rabbit IgG (Sigma Chemical Co., Poole, UK) in 100 µl of 0.1 mol/l sodium bicarbonate buffer, pH 9.6. Wells were then blocked with 0.5% bovine serum albumin in bicarbonate buffer. A competitive immunoluminometric assay was set up by preincubating 200 ng of the IgG with standards or samples within the wells. After overnight incubation, 50 µl of the diluted biotinylated ORP peptide tracer (2 µl /ml of the stock solution or a total amount of 100-500 fmol) was added to the wells. Following another 24 h of incubation at 4°C, wells were washed 3 times with a wash buffer (NaH_2PO_4 1.5 mmol/l, Na_2HPO_4 8 mmol/l, NaCl 340 mmol/l, Tween 0.5 g/l, sodium azide 0.1 g/l). Streptavidin labeled with methyl-acridinium ester (MAE) was synthesized as described [15]. Wells were incubated for 2 h with 100 µl of ILMA containing streptavidin-MAE (5 million relative light units per well). Following further washes, chemiluminescence was detected by sequential injections of 100 µL of 0.1 M nitric acid (with H_2O_2) and then 100 µL of NaOH (with cetyl ammonium bromide) in a Dynatech MLX Luminometer, The lower limit of detection (defined as 3 times standard deviation at zero peptide concentration) was 9.8 fmol per tube or 98 fmol/ml of plasma extracted. Within assay coefficients of variation were 3.1, 4.3 and 5.9% for 2, 30, 500 fmol/tube respectively. There was no cross-reactivity with peptides previously demonstrated to be elevated in heart failure such as ANP, BNP, N terminal proBNP or CNP

Assay N-BNP

The assay for N-terminal proBNP was based on the non-competitive N-terminal proBNP assay described by Karl [16]. Rabbit polyclonal antibodies were raised to the N-terminal (amino acids 1-12) and C-terminal (amino acids 65-76) of the human N-terminal proBNP. IgG from the sera was purified on protein A sepharose columns. The C-terminal directed antibody (0.5 µg in 100 µL for each ELISA plate well) served as the capture antibody. The N-terminal antibody was affinity purified and biotinylated. Aliquots (20 µL) of samples or N-BNP standards were incubated in the C-terminal antibody coated wells with the biotinylated antibody for 24 hours at 4°C. Following washes, streptavidin labeled with methyl-acridinium ester (streptavidin-MAE, 5×10^6 relative light units /ml) [15] was added to each well. Plates were read on a Dynatech MLX Luminometer as previously described [15]. The lower limit of detection was 5.7 fmol/ml of unextracted plasma. Within and between

assay coefficients of variation were acceptable at 2.3% and 4.8% respectively. There was no cross-reactivity with ANP, BNP or CNP.

Size exclusion chromatography and gel electrophoresis of plasma extracts

Plasma extracts were fractionated by isocratic size exclusion chromatography on a 300 x 7.8mm Bio-Sep SEC S2000 column (Phenomenex, Macclesfield, Cheshire, UK) using 50 mmol/l NaH_2PO_4 (pH 6.8) at a flow rate of 1 ml/min as the mobile phase. Standards used to establish molecular weights included IgG (150kD), BSA (68kD) , ovalbumin (44kD), soybean trypsin inhibitor (20kD), aprotinin (6.5kD) and tryptophan (204D) (from Sigma Chemical Co, Poole, UK.). Fractions collected every 20 sec were dried on a centrifugal evaporator before assaying for ORP150 as above.

Statistical analysis

Statistical analysis was performed using SPSS Version 11.0 (SPSS Inc, Chicago, MI). Data are presented as mean ± SEM or median (range) for data with non-Gaussian distribution, which were log transformed prior to analysis. For continuous variables, one-way analysis of variance (ANOVA) was used. The interaction of multiple independent variables was sought using the univariate General Linear Model procedure with least significant difference P values reported. Pearson correlation analysis was performed and box plots were constructed consisting of medians, boxes representing interquartile ranges and the whiskers representing the 2.5^{th} to the 97.5^{th} centile. P values below 0.05 were considered significant. Kaplan Meier survival analysis was used to examine the usefulness of peptide levels in risk stratification following MI.

Results & Discussion

Performance of the ORP150 assay

A typical standard curve for ORP150 peptide is illustrated in Figure 1, showing a fall in chemiluminescence with increasing concentrations of the peptide. Half displacement of binding of the tracer occurred at about 300 fmol per tube. Dilutions of a heart failure patients' plasma and urine extracts showed parallelism with the standard curve. The lower limit of detection was 9.8 fmol/tube.

In addition, isocratic size exclusion chromatography was performed on human plasma extracts (Figure 2). This was resolved into 3 main immunoreactive fractions, one at 150 kD (which is the expected molecular weight of human ORP150 protein), a smaller peak at 6.7 to 7.4 kD and the largest one at 1.8 to 3.3 kD. This suggests that ORP150 extracted from plasma is fragmented and there may be other fragments that could be detected with other epitope specific antibodies.

Conclusions on detection of ORP in humans

Specific immunoassays for ORP have detected the presence of this peptide in plasma and urine. As ORP150 is an endoplasmic reticulum associated protein, this finding is unexpected.

Moreover, the immunoreactivity in plasma is derived from several molecular weight forms, suggesting that fragments of ORP150 may be detectable using epitope specific antibodies.

ORP150 in Normal subjects, Heart Failure and Myocardial Infarction

The characteristics of the normal, heart failure (HF) and myocardial infarction (MI) patients are shown in Table 1. Groups were well matched for gender. The normal and HF groups were matched for age, although the MI group was older than the other groups ($P<0.001$). Peptide levels were normalised by log transformation before analysis. Figure 3 shows the N-BNP and the ORP150 levels in the normal, HF and MI patient groups. Using ANOVA, differences in Log N-BNP ($P<0.0005$) and Log ORP150 ($P<0.0005$) was evident between the 3 groups. For N-BNP, both the HF and MI patients' levels were higher than normal ($P<0.0005$ using Tukey's test for multiple comparisons), but levels in HF and MI groups were comparable (P not significant). For ORP150, both the HF and MI patients' levels were higher than normal ($P<0.0005$ using Tukey's test for multiple comparisons). Levels in the HF group were also significantly higher than those in the MI group ($P<0.0005$).

ORP150 in heart failure

Within the normal group, there were age dependent changes in N-BNP (correlation coefficient $r = 0.438$, $P<0.0005$). However, ORP150 was not significantly correlated with age. Combining the normal and HF groups, N-BNP was again correlated with age ($r = 0.306$, $P<0.0005$) whereas the correlation of ORP150 with age was modest ($r = 0.138$, $P<0.02$).

Figure 4 shows the N-BNP and ORP150 levels in normals and HF, for both gender. Levels of the peptides are elevated in both males and females with HF ($P<0.0005$ for both, using univariate general linear model (GLM) procedure). The elevation of both peptides in HF is dependent on the severity of HF as judged by the NYHA class. Figure 5 shows that both peptides rise with increasing NYHA class in both gender. For N-BNP, values in normal subjects were different from NYHA class I, II, III and IV ($P<0.0005$ for all using Tukey's test). For ORP150, values in normal subjects were different from NYHA class I, II, III and IV ($P<0.002$, 0.0005, 0.0005, 0.0005 respectively using Tukey's test).

Using the univariate GLM procedure, and entering age as a covariate and gender and NYHA class as factors, analysis of the log normalised N-terminal proBNP levels in the heart failure patients yielded an r^2 of 0.675 for the model ($P<0.0005$) with age, gender and NYHA class as significant predictive variables ($P<0.0005$ for all). There was a significant interaction between gender and NYHA class suggesting that the rise in N-BNP with increasing NYHA class may differ between males and females ($P<0.007$). A similar analysis performed on the log normalised ORP150 data yielded an r^2 of 0.512 for the model ($P<0.0005$) with NYHA class only as a significant predictive variable ($P<0.0005$). Age and gender were not significant predictive variables, although there was a significant interaction between gender and NYHA class ($P<0.001$) again suggesting that the rise in ORP150 with increasing NYHA class differs between males and females. Although the majority of HF patients have ischaemic heart disease as the aetiology, detection of HF using these peptides is achieved irrespective of aetiology.

Stepwise logistic regression analysis was employed to predict absence or presence of HF, with log N-BNP and log ORP150 as predictive variables. Age and gender were not used since the normal and HF groups were age and gender matched. Both N-BNP (Odds ratio for 50% rise in peptide level 1.56, $P<0.0005$) and ORP150 (Odds ratio for 50% rise in peptide

level 2.46, P<0.0005) were independent predictors of presence of HF, accounting for a total r^2 (Cox and Snell) of 0.55 and a Nagelkerke r^2 of 0.74 irrespective of whether forward or backward stepwise procedures was used. The prognostic index (probability of membership of HF group) derived from the above model was used to construct a receiver operating characteristic (ROC) curve (figure 6). The ROC area for the model was 0.95, greater than that of N-BNP (0.91) or ORP (0.84) alone, for the identification of HF.

Conclusions on ORP150 in Heart Failure

These findings suggest that although both N-BNP and ORP150 are elevated in HF (and with increasing severity of HF), N-BNP is more affected by age and gender of the subjects (with higher levels with rising age and in females). ORP150 by contrast does not have an age dependent component and is modestly affected by gender. Both peptides are effective in identification of HF, but the combination of the 2 may have added potential in diagnosis of HF.

ORP150 in myocardial infarction

The patient characteristics of the myocardial infarction (MI) group are shown in Table 1, and although gender matched were slightly older than the normal group (P<0.0005). Both N-BNP and ORP150 were elevated in the plasma obtained 2-3 days after myocardial infarction (P<0.0005 for both, figure 3). Levels of N-BNP were correlated with the peak creatine kinase level (r = 0.24, P<0.0005) suggesting a relation to the size of the infarction. However, ORP150 levels were not significantly correlated to the peak creatine kinase level (r = 0.05, P not significant).

N-BNP was correlated to both age (r = 0.39, P<0.0005) and creatinine (r = 0.38, P<0.0005), the partial correlation coefficients remaining significant after allowing for the effects of gender and infarction (with age (r = 0.39, P<0.0005) and with creatinine (r = 0.36, P<0.0005)). In contrast, ORP150 was not significantly correlated with age, but weakly with creatinine (r = 0.20, P<0.0005), the partial correlation coefficient falling further after allowing for the effects of gender and infarction (with creatinine (r = 0.12, P<0.007).

The determinants of log normalised ORP150 were sought using stepwise linear regression analysis with age and creatinine as covariates, and presence of MI and gender as factors. Only presence of MI (P<0.0005) and creatinine (P<0.004) were identified as significant independent predictors of ORP150 levels, accounting for 14% of total variance (P<0.0005). A similar analysis with N-BNP levels identified age, gender, creatinine and presence of MI as significant independent predictors (P<0.0005 for all). Thus this finding confirms that in the HF group, i.e. ORP150 levels are less susceptible to influence by age and gender than N-BNP levels.

We used logistic regression analysis to predict presence or absence of MI as the dependent variable, using age, gender, N-BNP and ORP150 as independent variables. All 4 were identified as independent predictive variables for presence or absence of MI using both forward and backward stepwise regression analysis, the model accounting for an r^2 of 0.56 (Cox and Snell) or 0.79 (Nagelkerke). The odds ratios were as follows:- for N-BNP (for a 50% rise in the peptide level 1.94, P<0.0005); for ORP150 (for a 50% rise in the peptide level 1.61, P<0.0005).

The plasma level of N-BNP was related to the Killip class of the patient (figure 7, P<0.0005). In contrast, levels of ORP150 were elevated in all MI patients irrespective of Killip class (figure 7). Of the 177 patients who had echocardiography scans, we found that the

N-BNP levels was related to degree of LV dysfunction (figure 8, P<0.0005). In contrast, ORP150 levels were elevated in all MI patients irrespective of degree of LV dysfunction and even patients who had apparently "normal" LV function had elevated ORP150 levels (figure 8).

Outcomes after MI

All cause mortality and readmission rates with heart failure following MI were examined, to investigate the usefulness of ORP150 in prediction of these outcomes. Mean length of follow-up after discharge was 426 days with a range of 5-764 days. Out of the 367 cases, there were 39 deaths during the follow up period. There were also 22 readmissions with heart failure.

Patients who died had significantly higher N-BNP and ORP150 levels (P<0.0005 and P<0.001 respectively, figure 9). In addition, both peptides were elevated in those patients who were later readmitted with heart failure (P<0.0005 for N-BNP, P<0.025 for ORP150, figure 10).

Logistic regression analysis was used to investigate the predictors of death as an outcome with age, creatinine, past medical history of infarction, Killip class, and log N-BNP or Log ORP150. Significant independent predictors for death included N-BNP (odds ratio for 10 fold rise in peptide level 3.95, P<0.002) and ORP150 (odds ratio for 10 fold rise in peptide level 4.58, P<0.05), accounting for a Nagelkerke r^2 of 0.32. Backward and forward regression analysis confirmed these two independent predictor variables, but with an additional contribution from creatinine (odds ratio for 10 fold rise 19.56, P<0.05). These findings suggest that ORP150 is a predictor of mortality after MI independent of N-BNP levels.

Kaplan Meier survival analysis was performed to confirm these findings. When subjects were divided in infra and supra median groups, survival differed significantly between these 2 groups (figure 11), whether the peptide used was N-BNP (P<0.0005 by log rank test for trend) or ORP150 (P<0.002 by log rank test for trend). Of note is that even in the infra median groups defined by N-BNP or ORP150 alone, there is a definite mortality rate (albeit slower than the supra median groups). We utilised the ranks in both the N-BNP and ORP150 ranked groups to yield a novel prognostic index, with patients divided up into 3 groups (both peptides below the medians, either peptide above the medians and both peptides above the medians). Figure 12 shows the survival analysis using this new prognostic index, showing no deaths during the observational period in the group with both peptides below the medians, a high mortality rate in those with both peptides above the medians, and an intermediate mortality rate in those with either peptide above the medians (P<0.0005 by log rank test for trend).

Conclusions on ORP in MI

Plasma ORP150 levels are elevated in ischaemic heart disease as manifested by myocardial infarction. In contrast to N-BNP which is also elevated in these patients, ORP150 levels are less dependent on age, degree of LV dysfunction, symptoms and signs (as determined by Killip class) and renal function. Both peptides are good predictors of outcomes such as mortality or readmission with heart failure following the index admission with myocardial infarction. In particular, the combination of both peptides may be particularly useful in risk stratification after myocardial infarction (prediction of mortality).

Overall Conclusions on ORP in vascular disease

The above data demonstrates that ORP150 is secreted into human plasma and can also be found in urine. There may be fragments of ORP150 in bodily fluids. The levels of ORP150 are elevated in both Heart Failure and Ischaemic Heart Disease, and the measurement may be less prone to age and gender interference. As atherosclerosis is the major cause of vascular disease, ORP150 may be of use in the diagnosis or prognosis of other conditions where there is tissue hypoxia, for example, strokes, peripheral vascular disease, aneurysms, or acute coronary syndromes. In Heart Failure, in addition to being a diagnostic aid in itself, it could complement the measurement of N-BNP. In Myocardial Infarction, it may serve as an indicator of prognosis, predicting both death and readmissions with heart failure. Independently or in combination with N-BNP, its measurement after myocardial infarction is an effective aid to risk stratification able to detect extremely low or high risk groups of patients. This may have impact in the planning of therapeutic options for patients.

References

1) McDonagh TA, Morrison CE, Lawrence A, et al. Symptomatic and asymptomatic left ventricular dysfunction in an urban population. Lancet 1997; 350: 829-833
2) Davies MK, Hobbs FDR, Davis RC, et al. Prevalence of left ventricular systolic dysfunction and heart failure in the Echocardiographic Heart of England Screening Study: a population based study. Lancet 2001; 358: 439-444
3) McDonagh TA, Robb SD, Murdoch DR, et al. Biochemical detection of left ventricular systolic dysfunction. Lancet 1998; 351:9-13
4) Hobbs FD, Davis RC, Roalfe AK, Hare R, Davies MK, Kenkre JE. Reliability of N-terminal pro-brain natriuretic peptide assay in diagnosis of heart failure: cohort study in representative and high risk community populations. Br Med J 2002; 324 : 1498-1502
5) Kuwabara K, Matsumoto M, Ikeda J, Hori O, Ogawa S, Maeda Y, Kitagawa K, Imuta N, Kinoshita T, Stern DM, Yanagi H, Kamada T. Purification and characterization of a novel stress protein, the 150-kDa oxygen-regulated protein (ORP150), from cultured rat astrocytes and its expression in ischemic mouse brain. J Biol Chem 1996 ; 271 : 5025-32
6) Tsukamoto Y, Kuwabara K, Hirota S, Ikeda J, Stern D, Yanagi H, Matsumoto M, Ogawa S, Kitamura Y. 150-kD oxygen-regulated protein is expressed in human atherosclerotic plaques and allows mononuclear phagocytes to withstand cellular stress on exposure to hypoxia and modified low density lipoprotein. J Clin Invest 1996 ; 98 : 1930-41
7) Ikeda J, Kaneda S, Kuwabara K, Ogawa S, Kobayashi T, Matsumoto M, Yura T, Yanagi H. Cloning and expression of cDNA encoding the human 150 kDa oxygen-regulated protein, ORP150. Biochem Biophys Res Commun 1997 ; 230 : 94-9
8) Bando Y, Ogawa S, Yamauchi A, Kuwabara K, Ozawa K, Hori O, Yanagi H, Tamatani M, Tohyama M. 150-kDa oxygen-regulated protein (ORP150) functions as a novel molecular chaperone in MDCK cells. Am J Physiol Cell Physiol 2000 ; 278 : C1172-82
9) Matsushita K, Matsuyama T, Nishimura H, Takaoka T, Kuwabara K, Tsukamoto Y, Sugita M, Ogawa S. Marked, sustained expression of a novel 150-kDa oxygen-regulated stress protein, in severely ischemic mouse neurons. Brain Res Mol Brain Res 1998 ; 60: 98-106
10) Tamatani M, Matsuyama T, Yamaguchi A, Mitsuda N, Tsukamoto Y, Taniguchi M, Che YH, Ozawa K, Hori O, Nishimura H, Yamashita A, Okabe M, Yanagi H, Stern DM, Ogawa S, Tohyama M. ORP150 protects against hypoxia/ischemia-induced neuronal death. Nat Med 2001; 7 : 317-23
11) Ozawa K, Kuwabara K, Tamatani M, Takatsuji K, Tsukamoto Y, Kaneda S, Yanagi H, Stern DM, Eguchi Y, Tsujimoto Y, Ogawa S, Tohyama M. 150-kDa oxygen-regulated protein (ORP150) suppresses hypoxia-induced apoptotic cell death. J Biol Chem 1999; 274 : 6397-404
12) Tsukamoto Y, Kuwabara K, Hirota S, Kawano K, Yoshikawa K, Ozawa K, Kobayashi T, Yanagi H, Stern DM, Tohyama M, Kitamura Y, Ogawa S. Expression of the 150-kd oxygen-regulated protein in human breast cancer. Lab Invest 1998; 78 : 699-706

13) Ozawa K, Tsukamoto Y, Hori O, Kitao Y, Yanagi H, Stern DM, Ogawa S. Regulation of tumor angiogenesis by oxygen-regulated protein 150, an inducible endoplasmic reticulum chaperone. Cancer Res 2001; 61 : 4206-13
14) Ozawa K, Kondo T, Hori O, Kitao Y, Stern DM, Eisenmenger W, Ogawa S, Ohshima T. Expression of the oxygen-regulated protein ORP150 accelerates wound healing by modulating intracellular VEGF transport. J Clin Invest 2001 ; 108 : 41-50
15) Ng LL, O'Brien RJ, Demme B, Jennings S. A non-competitive immunochemiluminometric assay for Cardiotrophin-1 detects elevated plasma levels in human heart failure. Clinical Science 2002; 102: 411-416
16) Karl, Borgya, Galluser, et al. Development of a novel, N-terminal-proBNP (NT-proBNP) assay with a low detection limit. Scand J Clin Lab Invest Suppl 1999;230:177-181.
17) Asahi H, Koshida K, Hori O, Ogawa S, Namiki M. Immunohistochemical detection of the 150-kDa oxygen-regulated protein in bladder cancer. BJU Int 2002 ; 90 : 462-6

Table 1. Patient characteristics in the study. Means [ranges] are reported.

	Normal Controls	**Heart failure patients**	**Myocardial Infarction patients**
Number	180 (59 (32%)female)	120 (35 (29%) female)	373 (95 (26%)female)
Age (years)	61.2 [26-81]	61.4 [20-87]	65.1 [32-95]
Drug therapy	None		
Diuretics	-	98	176
β blockers	-	47	283
ACE inhibitors	-	99	220
Aetiology of Cardiomyopathy			
Ischaemic	-	80	373
Dilated	-	29	-
Hypertensive	-	7	-
Valvular	-	4	-

We claim

1. The use of oxygen regulated protein ORP150 or peptide fragments thereof as a marker of tissue hypoxia or as a marker of a clinical syndrome attributable to tissue hypoxia in a mammalian subject.

2. The use according to claim 1 wherein the clinical syndrome attributable to tissue hypoxia is due to heart failure, ischaemic heart disease, atherosclerosis, ischaemic stroke, aortic aneurysm, peripheral vascular disease, lung disease or tumour growth.

3. The use of (ORP150) or peptide fragments thereof as a cardiac marker for use as a diagnostic tool in the determination of the presence or severity of heart failure or ischaemic heart disease in a mammalian subject.

4. The use of ORP150 or peptide fragments thereof according to claim 3 in combination with a further marker indicative of heart failure or ischaemic heart disease, said marker preferably being a natiuretic peptide.

5. The use of oxygen regulated protein ORP150 or peptide fragments thereof according to claim 4 wherein the natiuretic peptide is brain natiuretic peptide (BNP) or N-terminal probrain natriuretic peptide (N-BNP).

6. A method for the determination of the presence or severity of tissue hypoxia or of a clinical syndrome indicative of tissue hypoxia wherein a sample of bodily fluid from a mammalian subject is measured for the presence of ORP150 or peptide fragments thereof.

7. A method according to claim 6 wherein the clinical syndrome indicative of tissue hypoxia is heart failure or ischaemic heart disease.

8. A method according to claim 6 wherein the bodily fluid is plasma.

9. A method according to claims 6-8 wherein a diagnosis or prognosis is made based upon the result obtained compared to that obtained from a healthy individual or individuals.

10. A method according to claim 4 wherein the measurement is carried out by use of an immunoassay.

Figure 1.

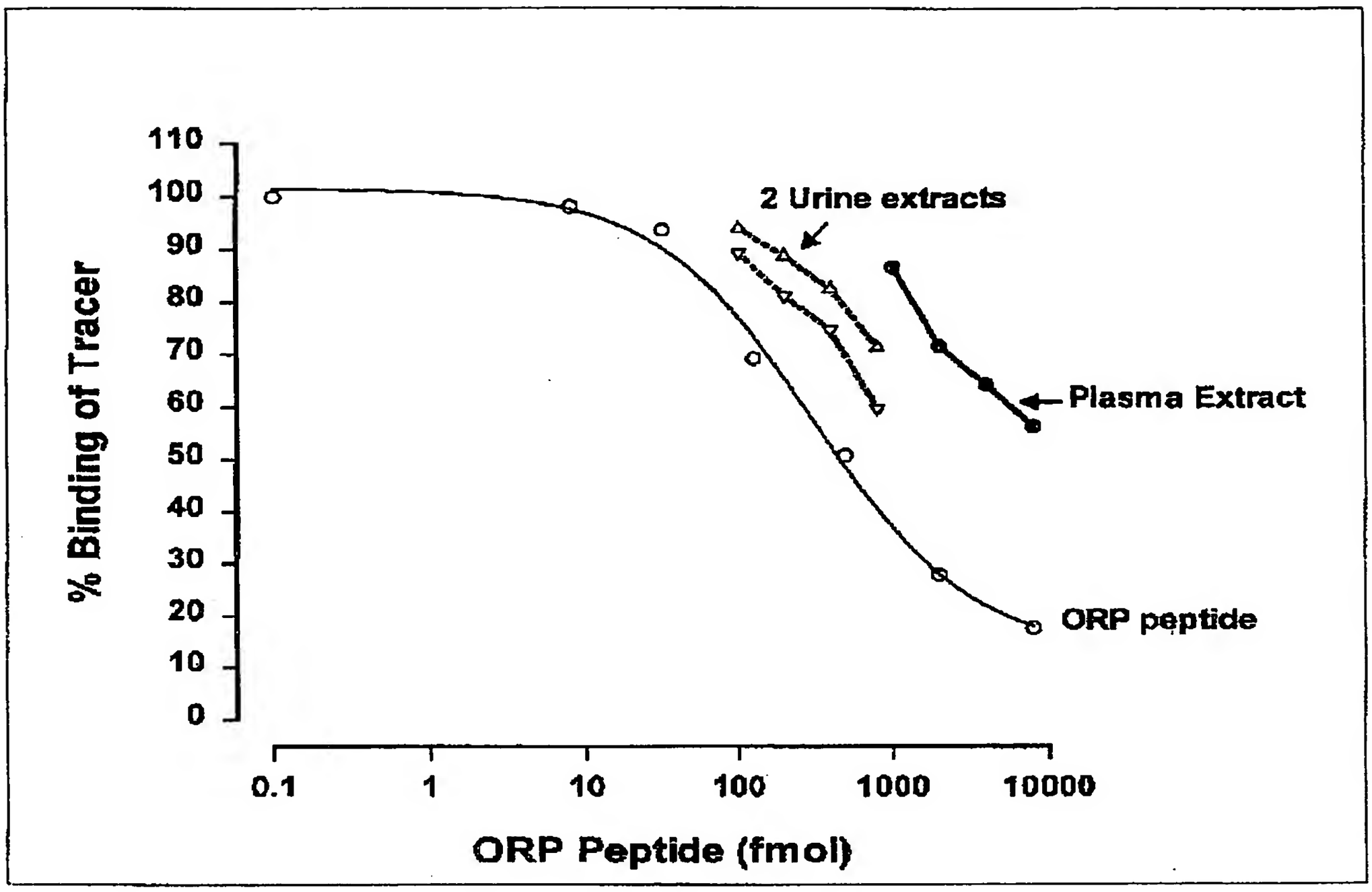


Figure 2

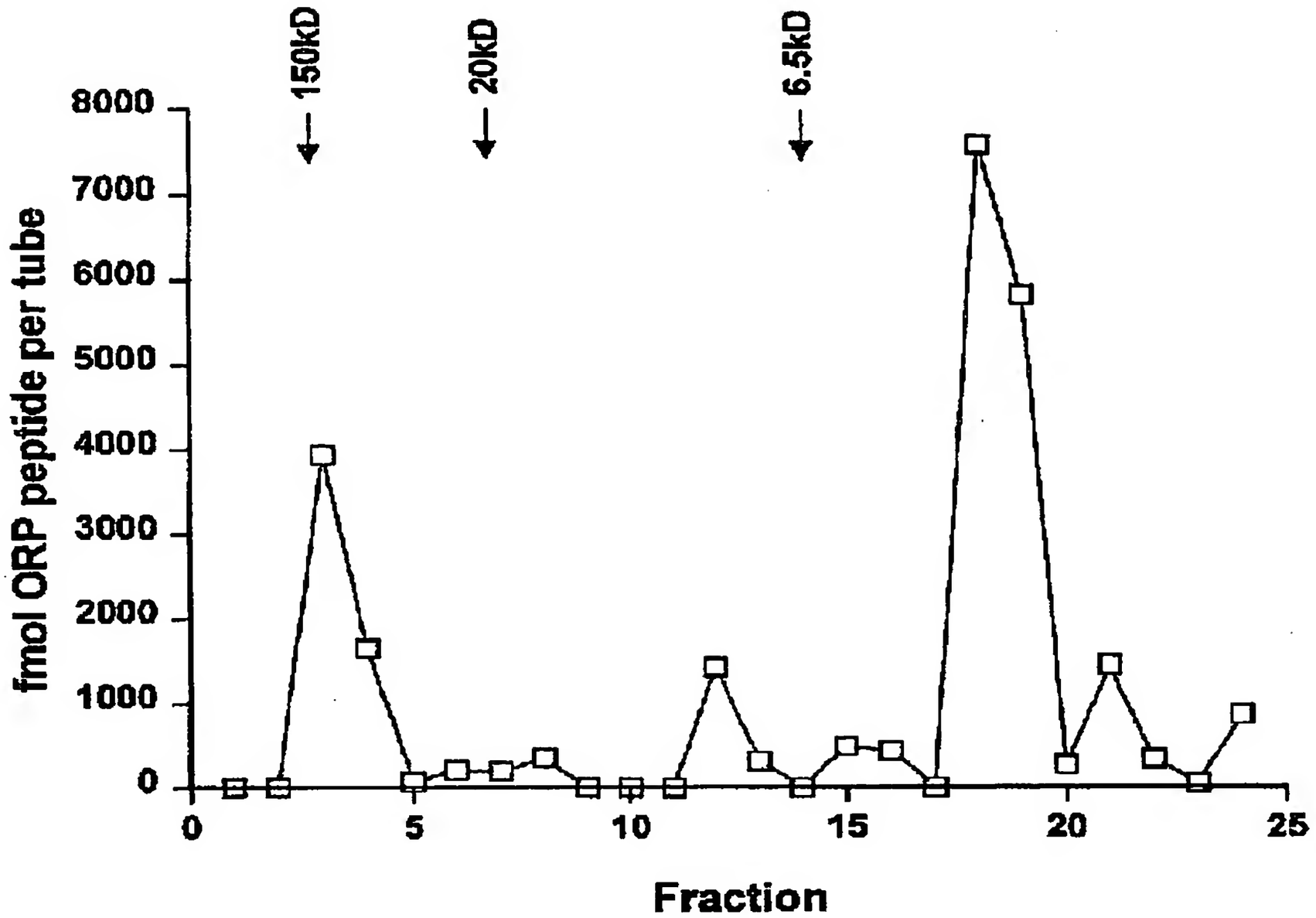
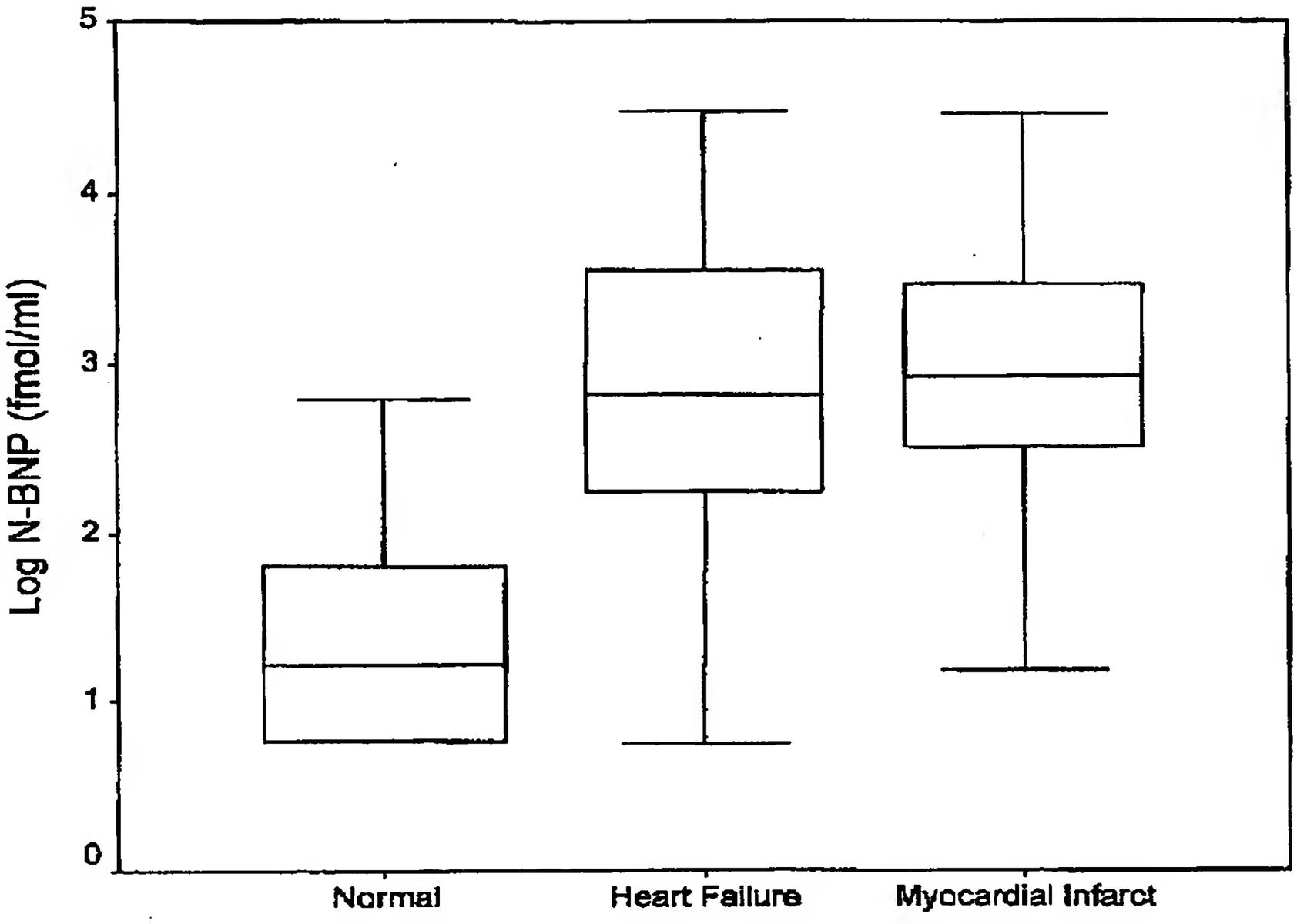


Figure 3



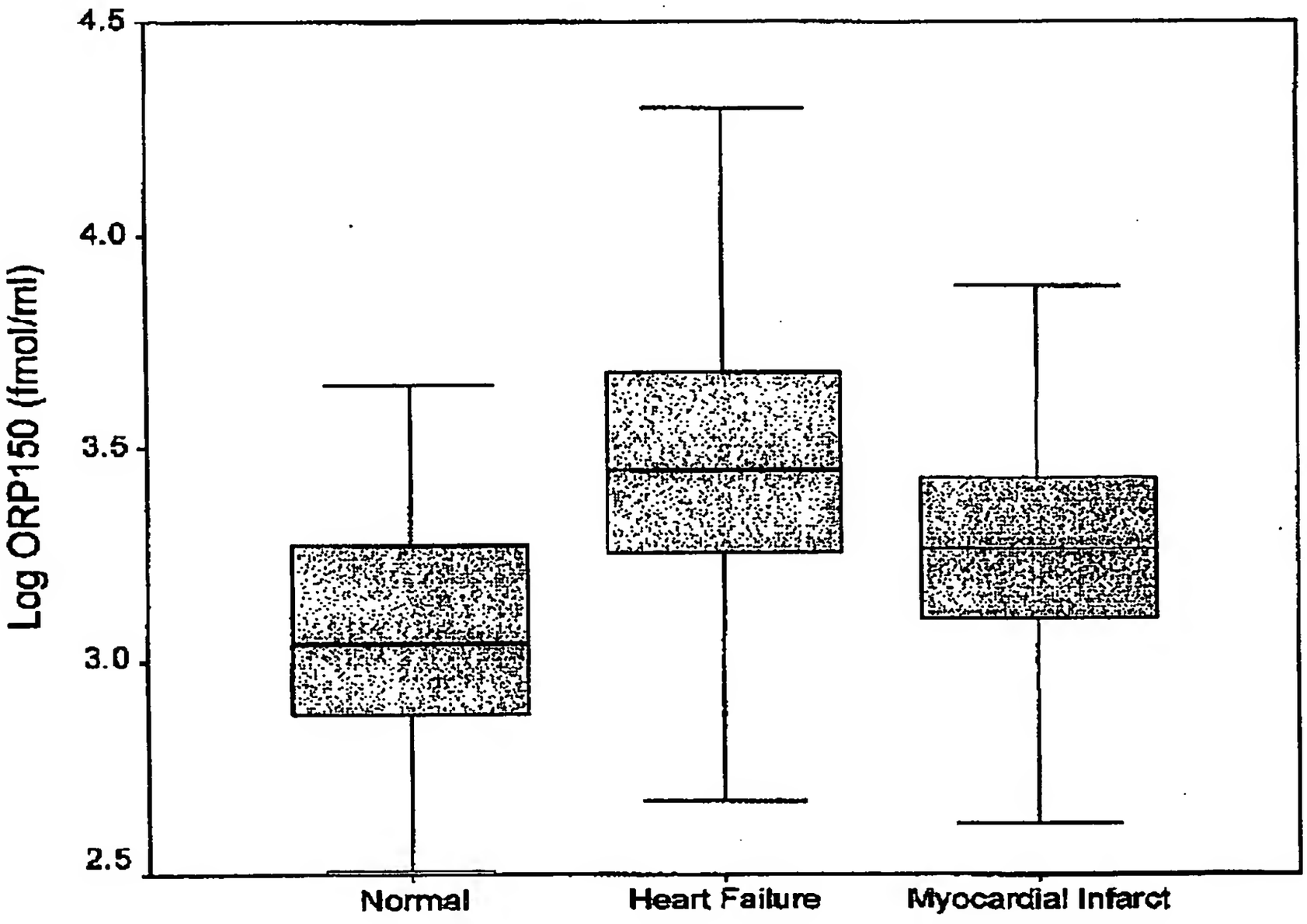
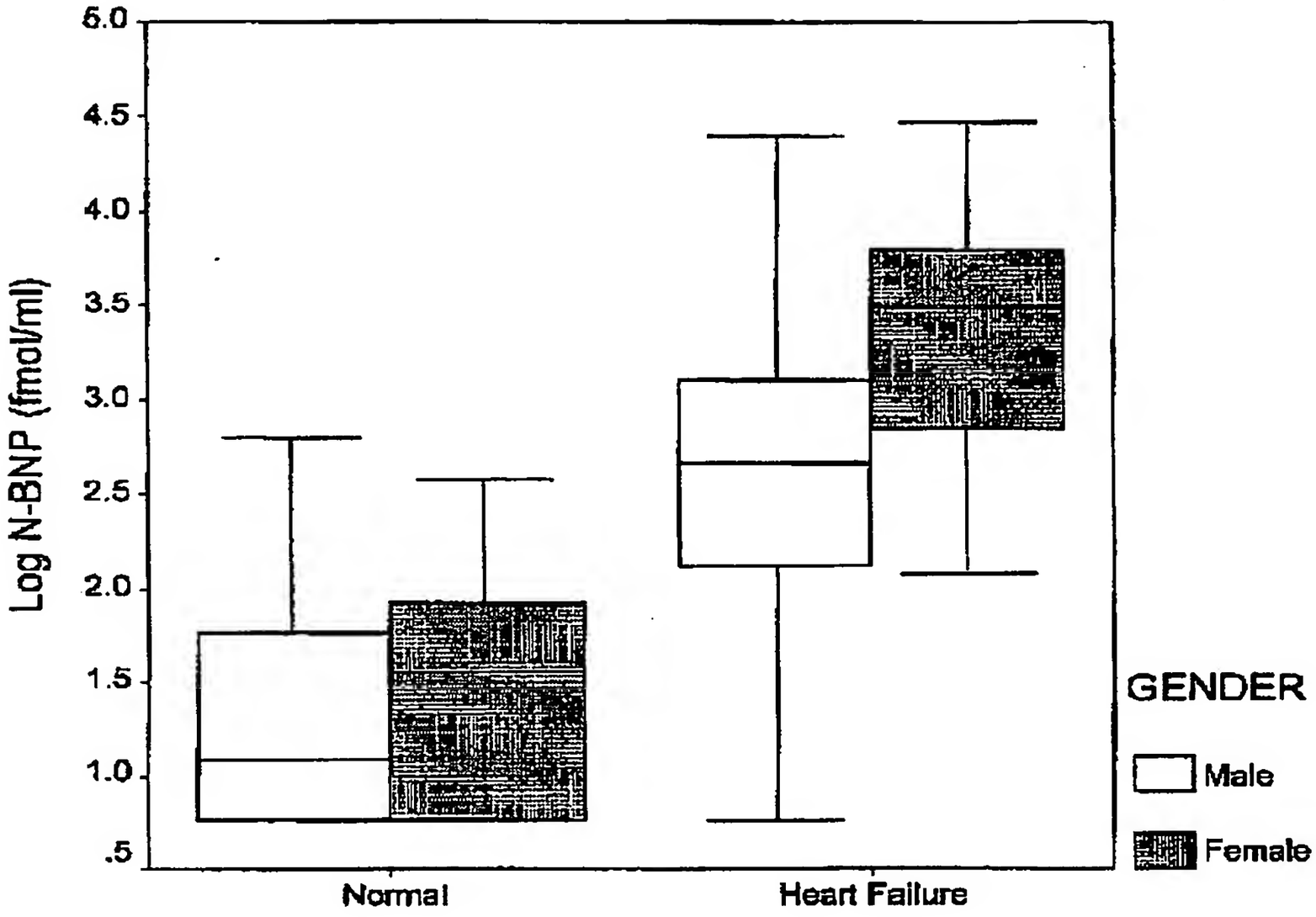


Figure 4



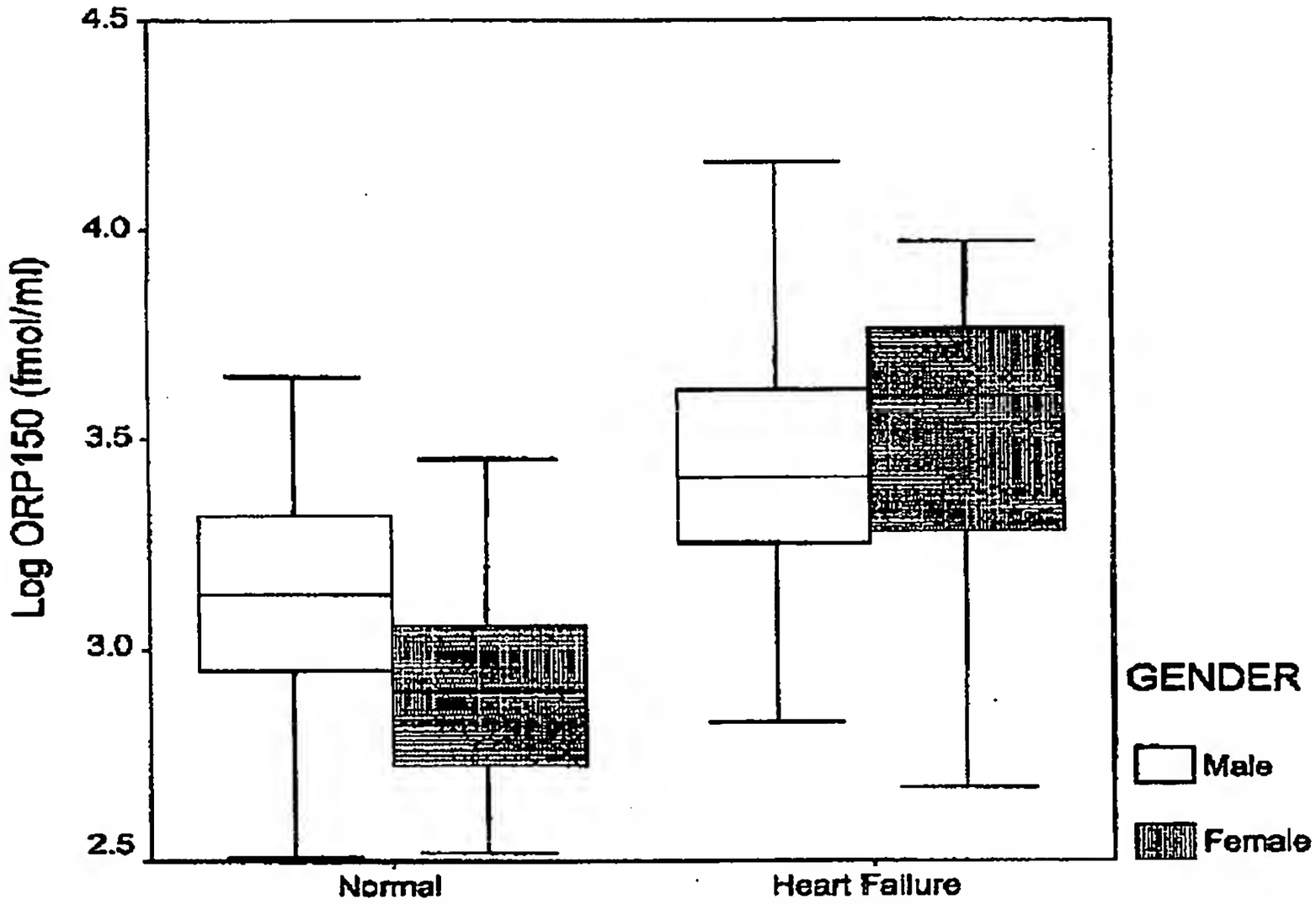
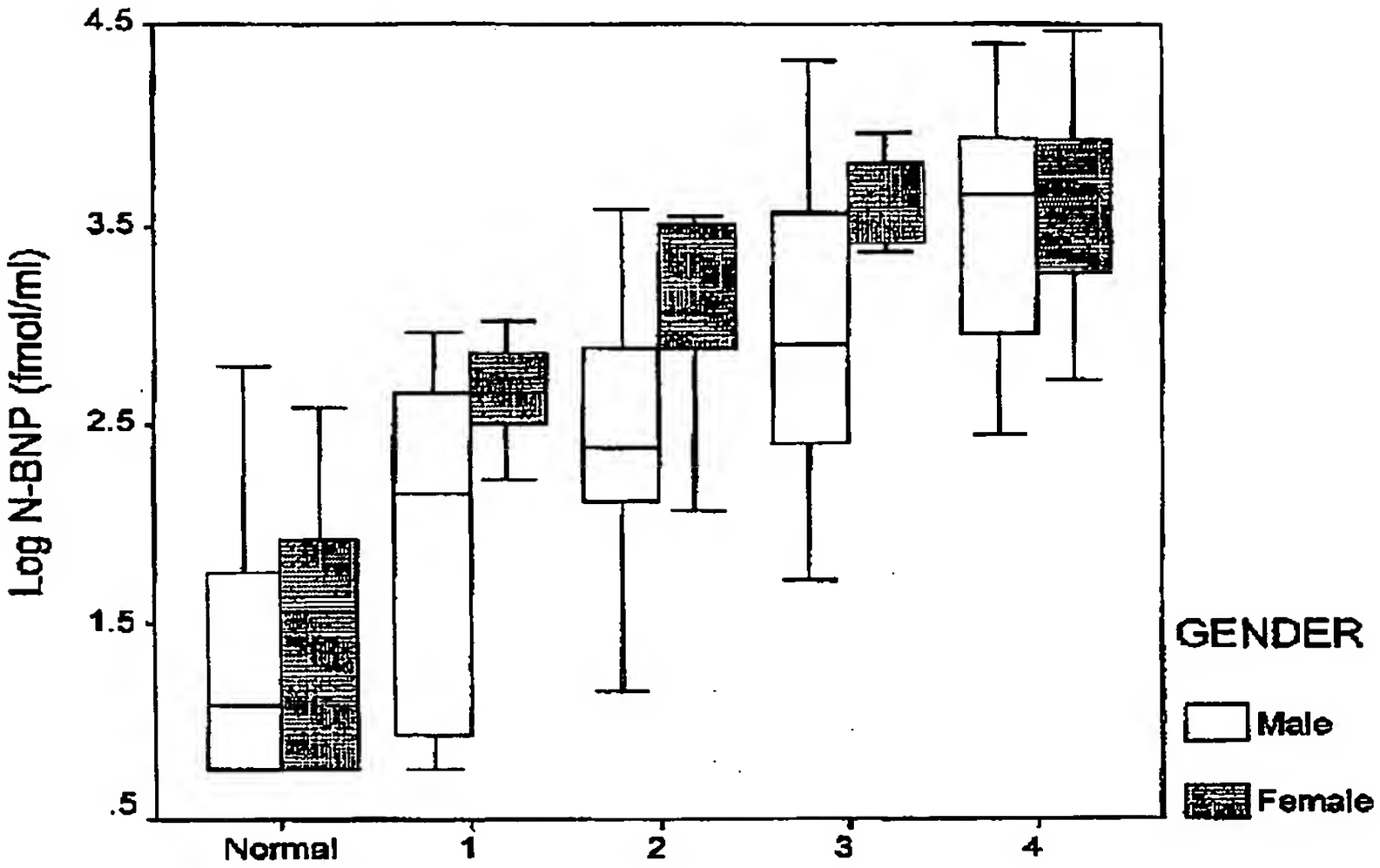


Figure 5



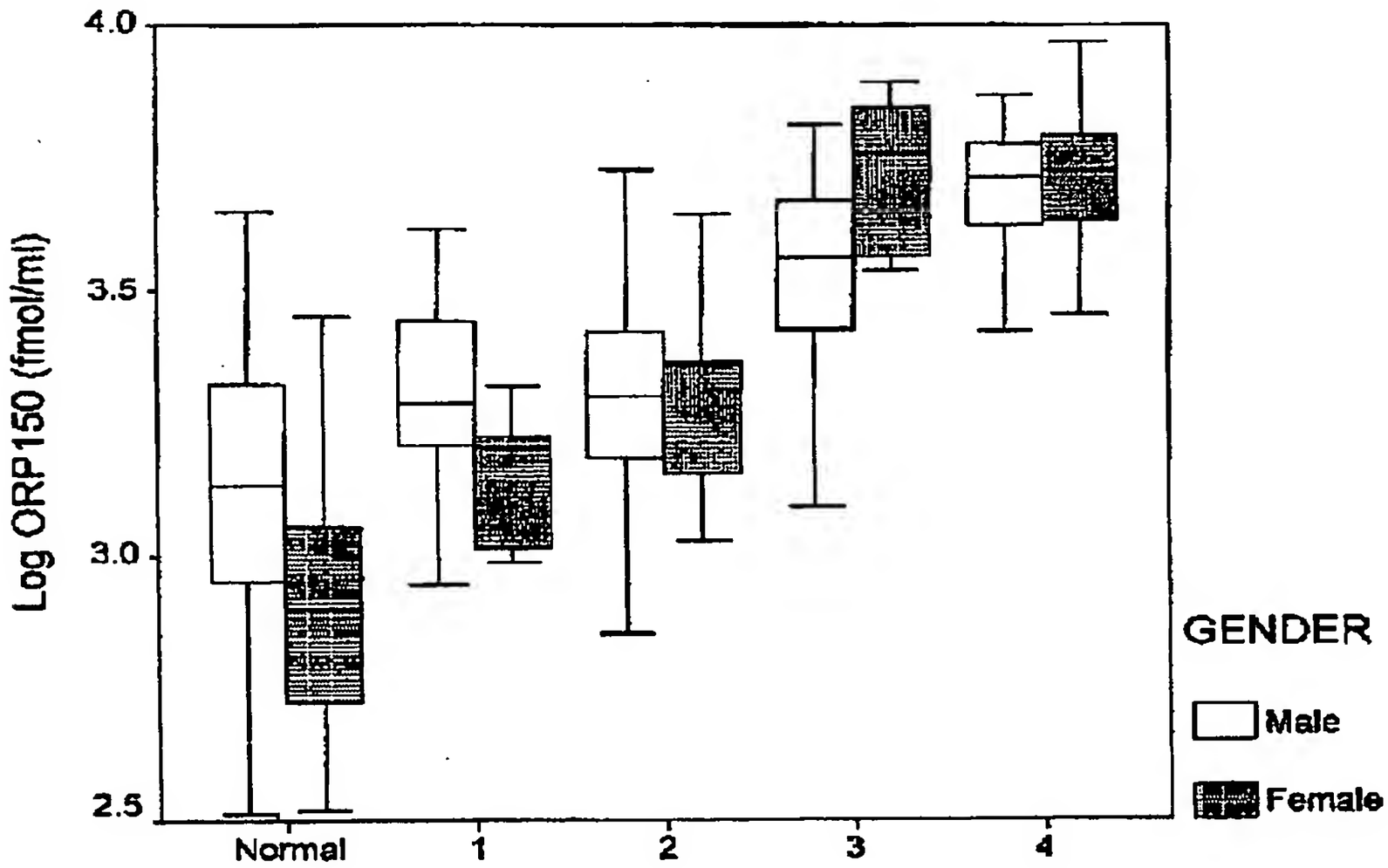


Figure 6

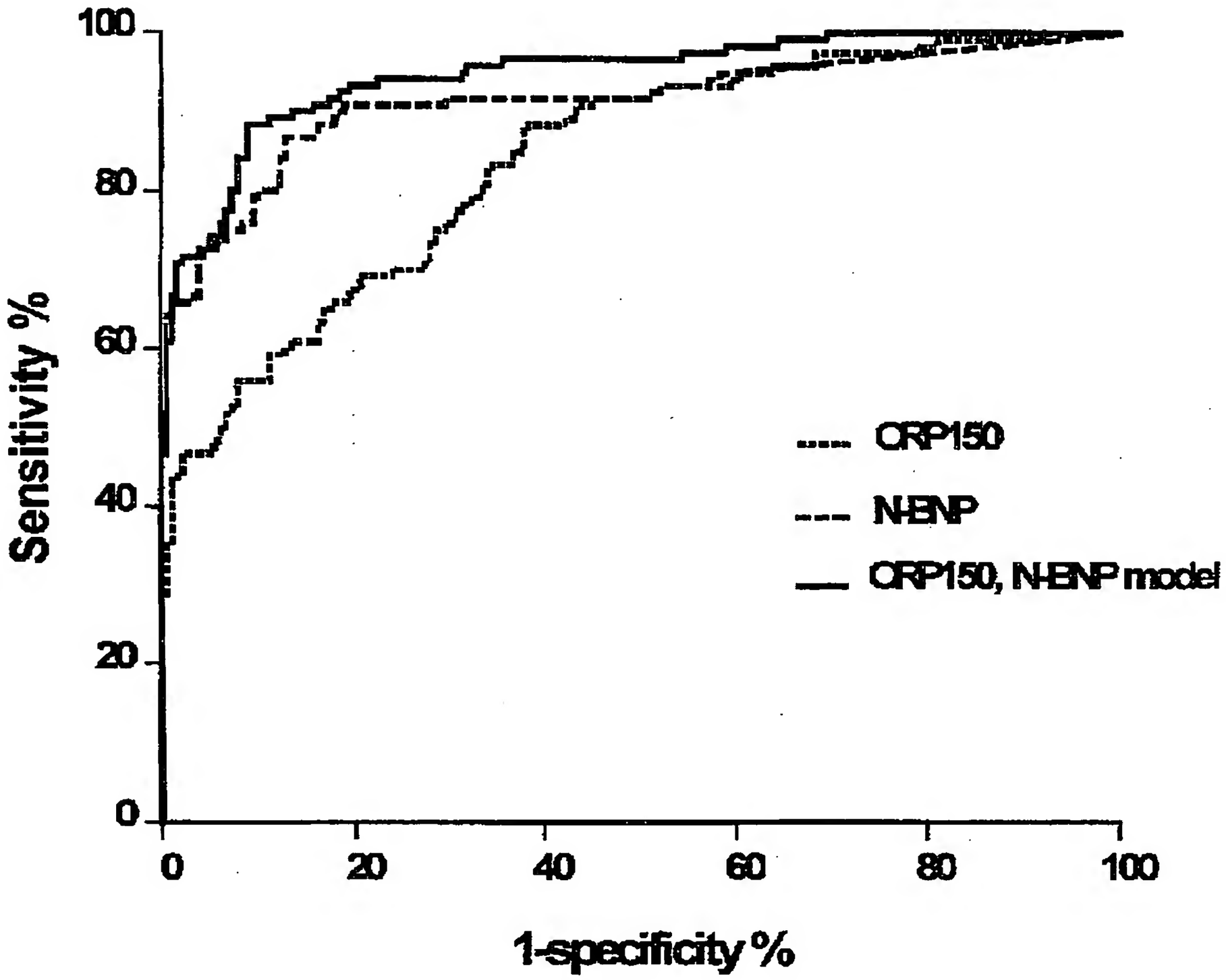

100
80
60
40
20
0
Sensitivity %
0
20
40
60
80
100
1-specificity %
CRP150
N-BNP
CRP150, N-BNP model

Figure 7

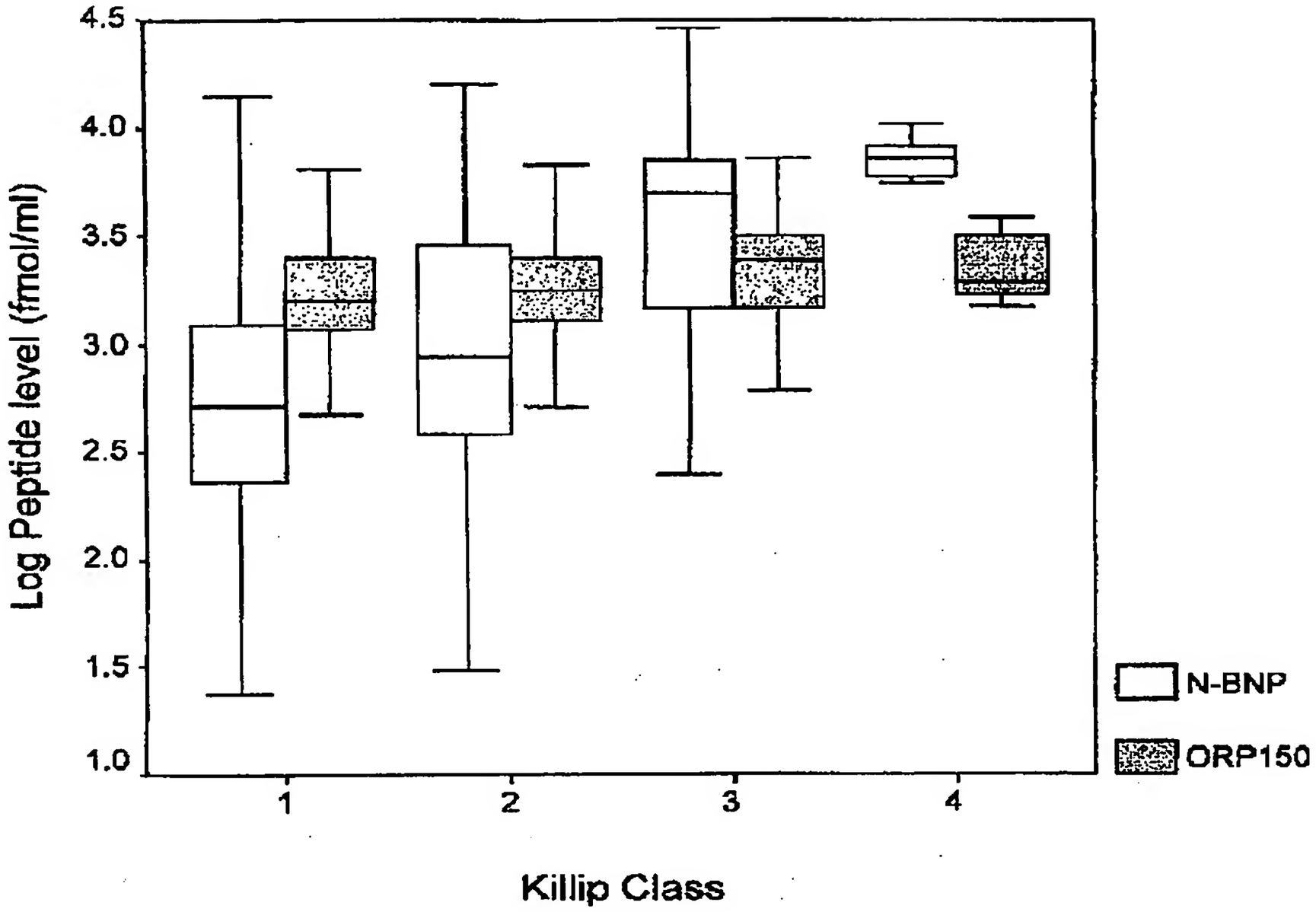


Figure 8

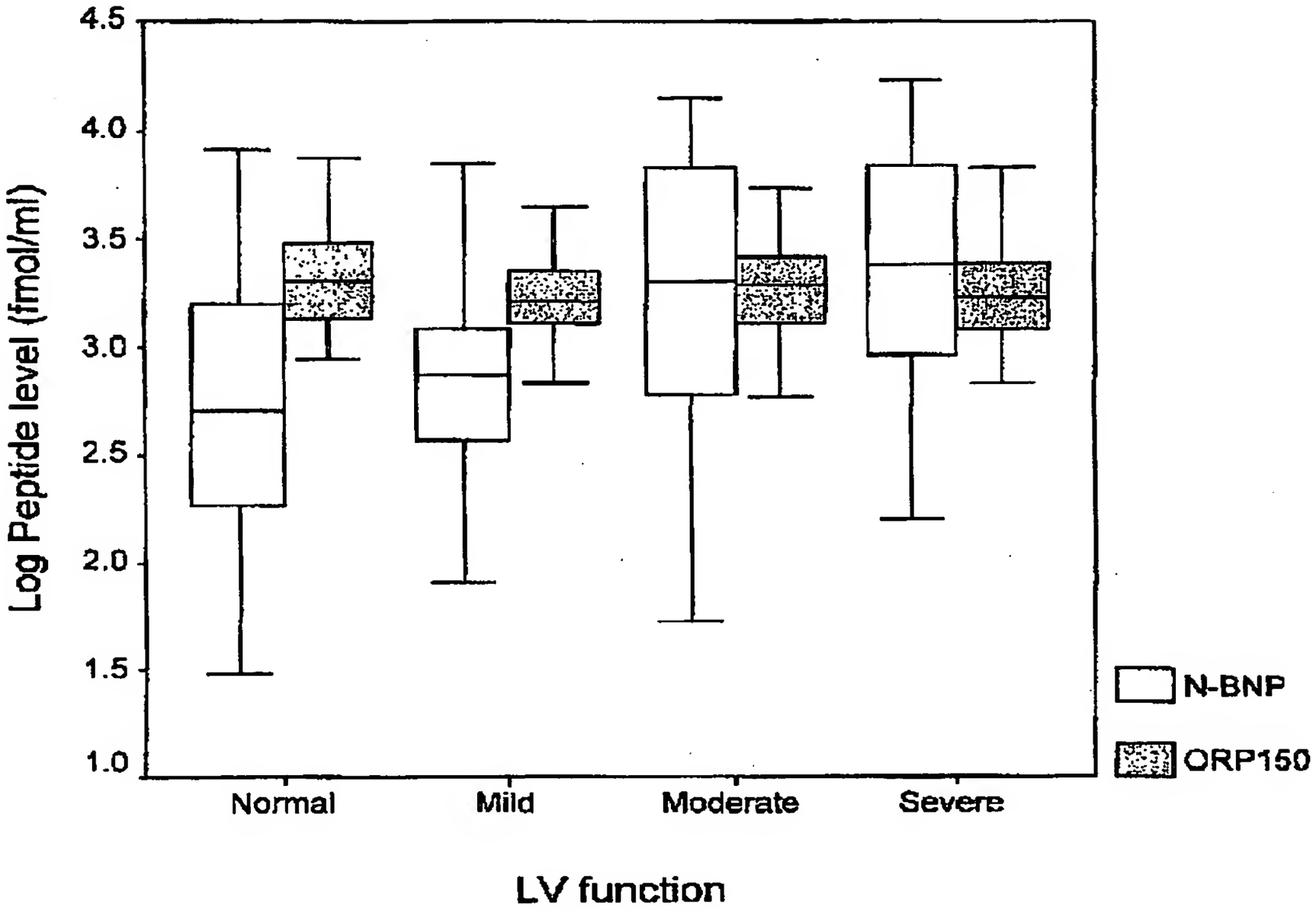


Figure 9

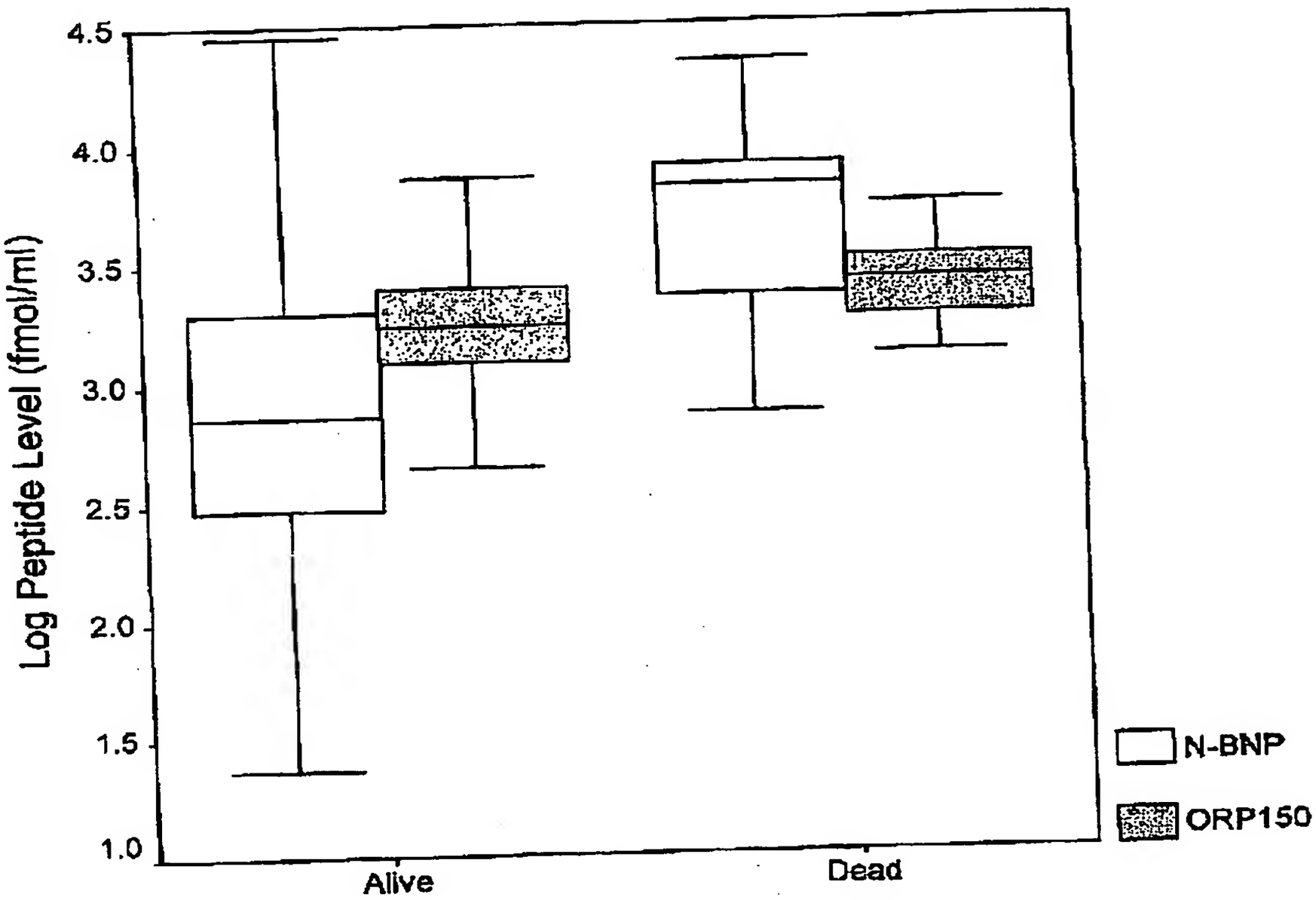


Figure 10

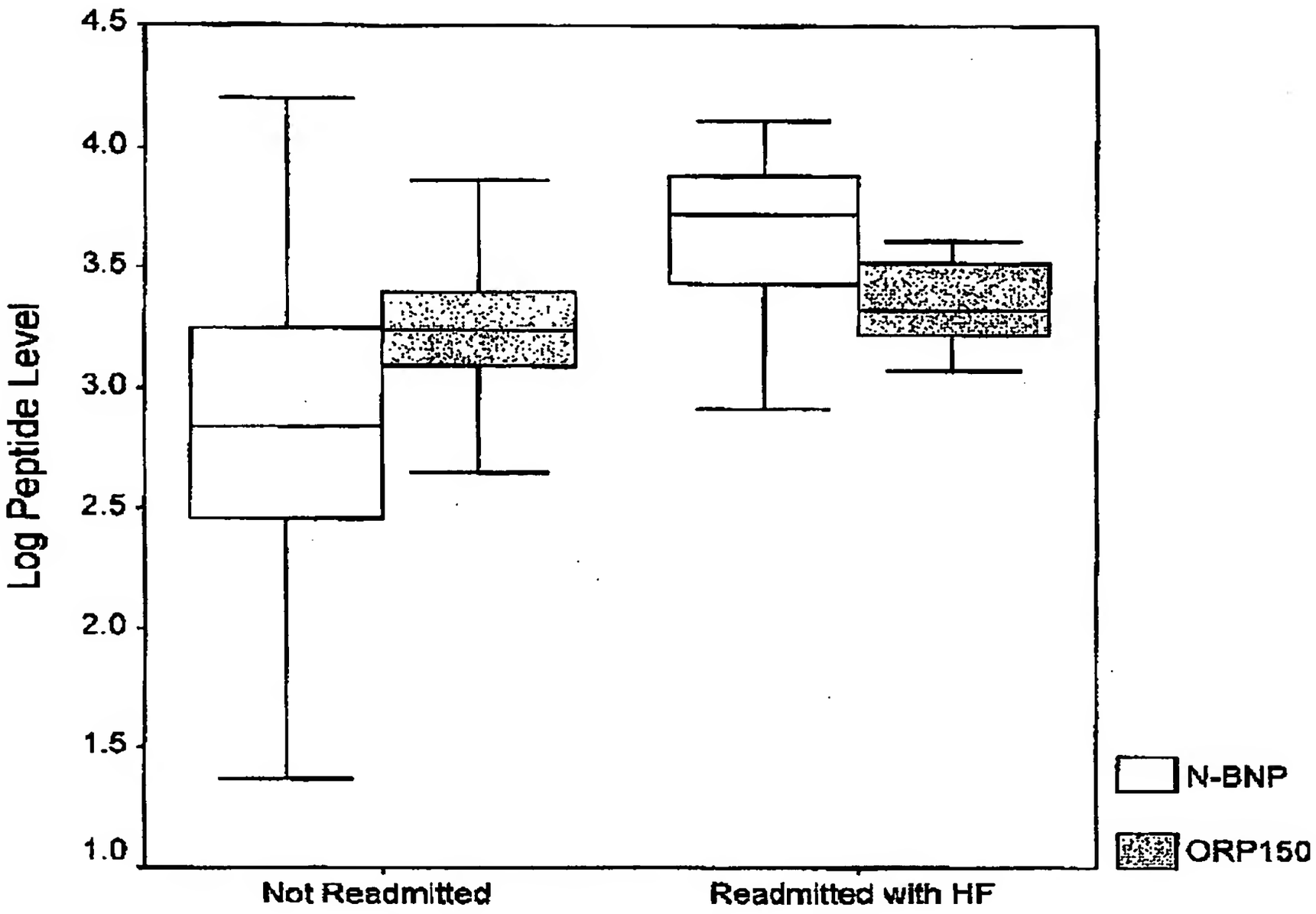
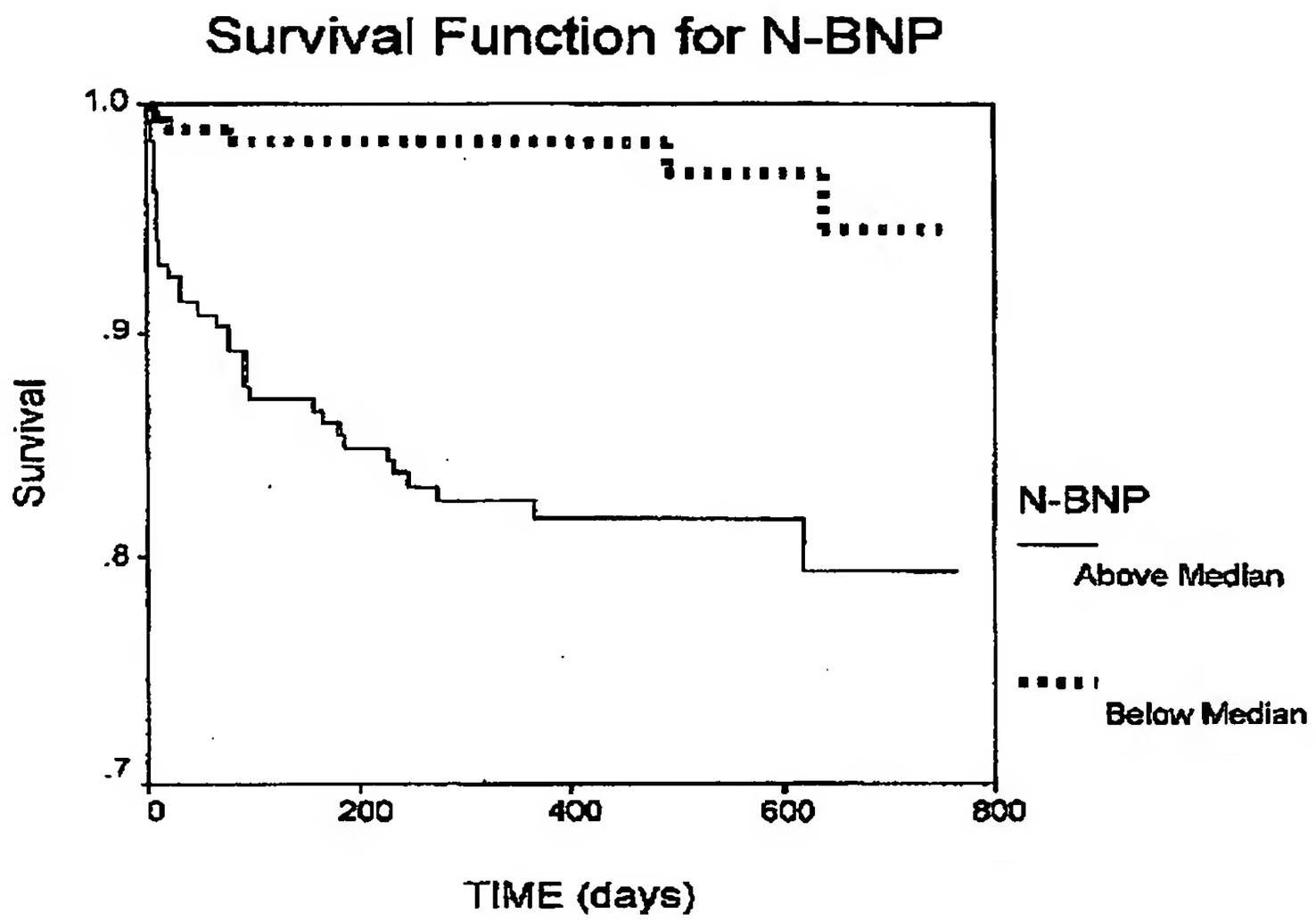

4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
Log Peptide Level
Not Readmitted
Readmitted with HF
N-BNP
ORP150

Figure 11



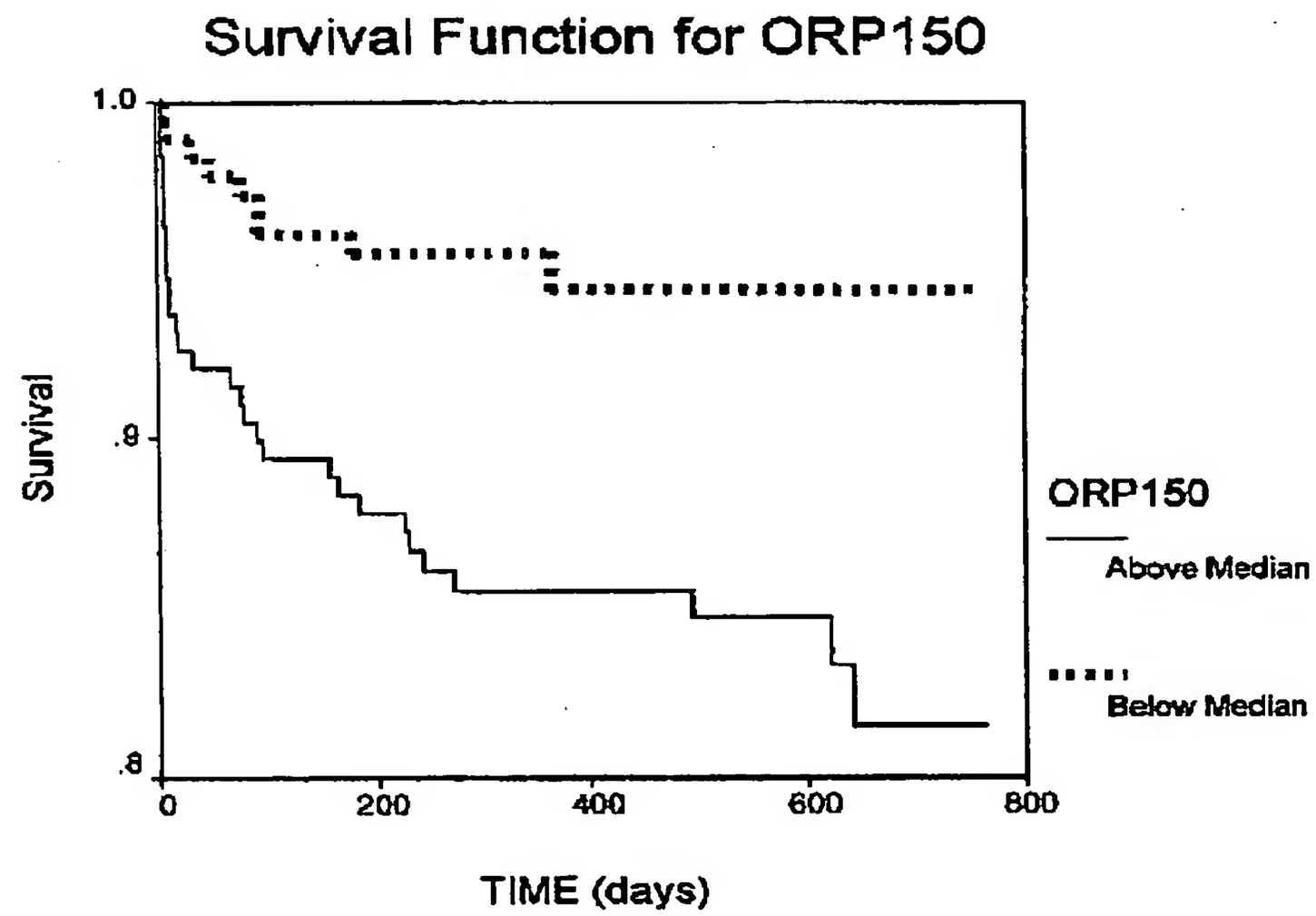


Figure 12